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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Che-Kun James Shen
Serial No. : 09/977,432
Filed : October 15, 2001
Title : HS-40 ENHANCER-CONTAINING VECTOR

Art Unit : 1636
Examiner : Kaushal Sumesh

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION BY CHEN-KUN JAMES SHEN UNDER 37 C.F.R. 1.132

I, Chen-Kun James Shen, hereby declare that:

1. I am the sole inventor of the subject matter described and claimed in the above-identified application, which relates to a viral expression vector having an enhancer that contains a mutant form of human HS-40 enhancer (SEQ ID NO: 1) disclosed therein.

2. In a final Office Action dated August 9, 2004 and an Advisory Action dated October 26, 2004, the Examiner maintained an obviousness rejection of all pending claims over Zhang et al., J. Biol. Chem. 270(15): 8501-8505 ("Zhang") in view of one or both of Miller et al., Biotechniqus 7(9): 980-990, 1989 ("Miller") and Jarman et al., Mol. Cell. Bio. 11(9): 4679-4689 ("Jarman"). According to the Examiner, (i) Zhang teaches a non-viral expression vector that has a SEQ ID NO:1-containing HS40 enhancer, (ii) Miller teaches retroviral vectors containing promoters, and (iii) Jarman teaches a regulatory element of the human α globin gene. As such, he concluded that it would be obvious to one skilled in the art to combine all cited references to make the claimed vector.

3. I or others have generated two viral expression vectors that contain the above-mentioned SEQ ID NO: 1 and its wild type counter part enhancer, respectively. In each vector, the enhancer is operatively linked to a ζ globin promoter and the growth hormone open reading frame.

The two just-described vectors were used to generate transgenic mice by standard methods. A total of 9 founders with the wild type vector ("wtHS40-ζGH") and 10 founders with the mutant vector ("mtHS40-ζGH") were obtained. The copy numbers of integrated fragments in the mice ranged from 1 to

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I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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more than 100. These transgenic mice were analyzed for blood GH protein level according to the method described in the actual example of the present specification.

It was found that the GH protein levels in adult wtHS-40 transgenic mice were all low and comparable to those in non-transgenic controls, indicating that the human ζ-globin promoter activity was essentially shut off in adult transgenic mice even when it was linked in cis with the wtHS-40 enhancer. In contrast, the blood GH levels of the mtHS-40 transgenic mice exhibited a roughly linear, positive relationship relative to transgene copy number. Further, all mice having similar transgene copy numbers exhibited similar levels of expression. This result indicates that the transgene expression in the mtHS-40 transgene is integration site-independent, i.e., position-independent, since the integration sites were random.

It was also found that the blood GH mRNA levels could not be detected in adult mtHS-40 transgenic mice unless the mice were first rendered anemic, indicating that expression was erythroblasts-specific. Control wtHS-40 transgene mice expressed little blood GH mRNA even rendered anemic. It was further found that wtHS-40 transgenic mice exhibited the expected temporal pattern of expression during development: the level of ζ-GH transcripts was relatively high at the 9.5-day embryo stage but dropped significantly in the adult blood. In contrast, the mtHS-40 transgenic mice continued to express the ζ-GH transcript into adulthood. In addition, even with only one copy of the transgene, mtHS-40 transgenic mice expressed a higher level of GH than wtHS-40 transgenic mice, regardless of the stage of development. These results indicate that transgene expression in the mtHS-40 transgenic mice was development stage-independent.

4. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully Submitted,

Date: 2004.11.08

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In Vivo Silencing of the Human γ-Globin Gene in Murine Erythroid Cells Following Retroviral Transduction

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(Communicated by Y. Kan, M.D., 11/14/00)

Hui-yu Lung,[1] Ilse S. Meeus,[1] Rona S. Weinberg,[1] and George F. Atweh[1]

ABSTRACT: Increased expression of fetal hemoglobin can ameliorate the clinical severity of sickle cell disease. Whereas temporary induction of fetal hemoglobin can be achieved by pharmacologic therapy, gene transfer resulting in high-level expression of the fetal γ-globin gene may provide a permanent cure for sickle cell disease. We had previously developed a high-titer, genetically stable retroviral vector in which the human γ-globin gene was linked to HS-40, the major regulatory element of the human α-globin gene cluster. Based on experience in transgenic mice, the truncated promoter of the γ-globin gene of this vector should be active in adult erythroid cells. Our earlier studies demonstrated that this retroviral vector can give rise to high-level expression of the human γ-globin gene in murine erythroleukemia (MEL) cells. We have now utilized this vector to transduce murine bone marrow cells that were transplanted into W/W^{v} recipient mice. Analysis of transduction of murine BFU-e's *in vitro* and peripheral blood cells from transplanted mice *in vivo* demonstrated efficient transfer of the human γ-globin gene. However, in contrast to the high level of expression of the human γ-globin gene of this vector in MEL cells, the gene was completely silent *in vivo* in all transplanted mice. These observations confirm that all the necessary regulatory elements responsible for the developmental stage-specific expression of the human γ-globin gene reside in its proximal sequences. They also emphasize the differences between gene regulation in MEL cells, transgenic mice, and retroviral gene transfer vectors. For this form of globin gene therapy to succeed, the proximal regulatory elements of the human γ-globin gene may have to be replaced with different regulatory elements that allow the expression of the γ-globin coding sequences in adult red cells *in vivo*.

INTRODUCTION

Although significant progress has been made in the treatment of sickle cell disease during the last decade, none of the currently available therapies are entirely satisfactory. Pharmacologic therapy with hydroxyurea, an agent that increases fetal hemoglobin (Hb F) production, has been shown to ameliorate the clinical severity of sickle cell disease (1). However, the long-term effects of treatment with hydroxyurea on the natural history of the disease and on overall survival remain to be determined. There are also lingering concerns about potential side effects that may result from life-long administration of a chemotherapeutic agent like hydroxyurea that inhibits repair of damaged DNA (2). These concerns have stimulated the search for safer agents with similar Hb F inducing activities such as butyrate and other short chain fatty acids (3). However, all agents that induce Hb F production, including hydroxyurea and butyrate, have transient activities and require life-long administration to maintain their therapeutic benefits. Other forms of experimental therapies that interfere with sickle red blood cell adhesion to the endothelium (4) or prevent the dehydration of sickle red blood cells (5) are currently under investigation. Bone marrow transplantation has recently been shown to provide a curative form of therapy for patients afflicted with this disease (6). However, although cures can be achieved with bone marrow transplantation, the

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procedure is still associated with significant morbidity and mortality (6). In addition, more than two thirds of sickle cell patients do not have appropriate siblings that could serve as bone marrow donors (6). Thus, in spite of these important therapeutic advances, there is still a need for safe and effective forms of curative therapies that will be available to all patients with sickle cell disease.

Efficient globin gene transfer into hematopoietic stem cells has been an elusive goal for many years (7–10). In the β-thalassemia syndromes where mutations of the β-globin gene result in decreased or total lack of production of adult β-globin chains, appropriate high-level expression of a transferred β-globin gene in the erythroid progeny of transduced stem cells should completely compensate for the genetic defect. The gene therapy of sickle cell disease is more challenging since the pathophysiology of the disease results from the accumulation of mutant β^S-globin chains rather than from a deficiency of production of normal β-globin chains. Although expression of normal β-globin chains in sickle erythrocytes may dilute the mutant β^S-hemoglobin and decrease the tendency of the red blood cells to sickle, expression of fetal γ-globin chains will probably provide more effective therapy because Hb F has better anti-sickling activity than Hb A (11).

Thus, in an attempt to develop gene therapy for sickle cell disease, we had generated retroviral globin vectors in which the human γ-globin gene was linked to HS-40 (also known as αLCR) (12). The use of HS-40 to enhance γ-globin expression solved the problems of genetic instability that result from the presence of elements of the βLCR in globin retroviral vectors (8, 13). We had previously shown that these γ-globin vectors are genetically stable and can be produced at high titers (12). Integration of these retroviruses in the genome of mouse erythroleukemia (MEL) cells gave rise to high-level expression of the human γ-globin gene in transduced cells (12). In this report, we describe our investigation of the performance of these retroviral vectors *in vivo* in a murine model of bone marrow transplantation.

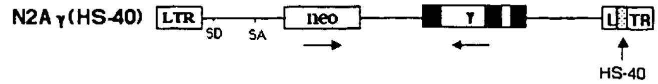

FIG. 1. Schematic illustration of the N2Aγ(HS-40) retroviral construct. The direction of transcription of the γ-globin gene and the neo^r gene are indicated by arrows. LTR, long terminal repeats; SD, splice donor; SA, splice acceptor.

MATERIALS AND METHODS

Transduction of Murine Progenitor Cells

The previously described retroviral vector N2Aγ(HS-40) (12) (Fig. 1) was used to transfer the human γ-globin gene into murine hematopoietic stem cells. C57BL/6J donor mice were given intravenous injections of 5-fluorouracil (150 mg/kg) to mobilize hematopoietic progenitor and stem cells (14). Three days later, the mice were sacrificed and their tibias and femurs were gently flushed with DMEM to harvest bone marrow cells. The harvested cells were prestimulated for 2 days in culture in the presence of murine recombinant IL-3 (40 ng/ml), human IL-6 (40 ng/ml) and murine stem cell factor (SCF) (40 ng/ml) as described (15). This was followed by cocultivation with ecotropic N2Aγ(HS-40) producer cells (12) for 2 days in the presence of polybrene (8 μg/ml) and the same growth factors. The producer cell line that was used in these experiments generated retroviral stocks whose titer exceeded 1×10^6 pfu/ml (12). Nonadherent cells were harvested, washed and plated at a concentration of 2×10^5 cells/ml in methylcellulose in the presence of 2 U/ml recombinant mouse erythropoietin as previously described (17, 18). Single BFU-e's were picked with a Pasteur pipet, lysed in proteinase K buffer and analyzed for the presence of the human γ-globin gene by a polymerase chain reaction (PCR) assay. The primers used for amplification of the human γ-globin sequences were 5′-TCACCCCTGGACATACTTTGCC-3′ and 5′-GGAAGATGCTGGAGGAGAAACC-3′. Each PCR (100 μl) included a single BFU-e colony suspended in PCR buffer with 30 pmol of each primer, 200 μM of the four dNTPs and 2.5 U of *Taq* polymerase. Amplifications were performed for 35 cycles, each consisting of denatur-

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ation for 1 min at 94°C, annealing for 1 min at 55°C and extension for 2 min at 72°C. Aliquots of the PCRs were analyzed by Southern blotting for the presence of an 890-bp amplified product corresponding to human γ-globin gene sequences.

Transduction of Murine Hematopoietic Stem Cells

Bone marrow cells were harvested from 5-fluorouracil-treated C57BL/6J donor mice and transduced with the ecotropic N2Aγ(HS-40) retroviral vector by cocultivation as described above. N2Aγ(HS-40)-transduced bone marrow cells from 40 donor mice (C57BL/6J) were selected in the presence of G418 (active concentration of 250 μg/ml) for 2 days before injection of the pooled selected cells into the tail veins of 4 unirradiated W/W^v recipient mice. Each W/W^v mouse was injected with approximately 9×10^6 selected bone marrow cells that were suspended in 0.3 ml of PBS. Blood was drawn from the retroorbital venous plexus of each transplanted mouse once a month for analysis of engraftment and assessment of integration and expression of the human γ-globin gene in peripheral blood cells. Engraftment was assessed by the change in electrophoretic mobility of globin chains of recipient mice from a W/W^v pattern to a C57BL/6J pattern as previously described (19). The presence of the human γ-globin gene in nucleated peripheral blood cells was assessed by a semiquantitative PCR assay in which the standards consisted of dilutions of DNA isolated from a MEL cell clone that carries a single integrated copy of the human γ-globin gene. Expression of the transduced gene was analyzed at the mRNA level by an S1 nuclease assay and at the protein level by an immunofluorescence assay as previously described (21).

RESULTS

Gene Transfer into BFU-e Colonies

Although the efficiency of transduction of BFU-e colonies does not generally predict the efficiency of gene transfer into hematopoietic stem cells, analysis of the efficiency of gene transfer into BFU-e colonies could be useful for confirming retroviral titers. We performed PCR amplification of the human γ-globin gene in 8 BFU-e colonies that were derived from plating a small aliquot of the same transduced bone marrow cells that were injected into recipient mice. Since the amount of DNA that is liberated by lysis of each BFU-e is very small and highly variable, we used Southern blot analysis to enhance the ability to detect the amplification products. The probe that was used in the Southern blotting experiment consisted of a 0.9-kb *Bam*HI/*Eco*RI fragment of the human γ-globin gene. Five of the eight colonies that were analyzed showed the presence of the expected 890-bp fragment (Fig. 2). This translates into an efficiency of transduction of BFU-e of 62.5%.

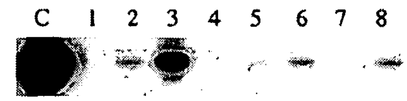

FIG. 2. PCR assay for the presence of human γ-globin sequences in murine BFU-e's. Lane C represents mouse DNA containing a single copy of the human γ-globin gene. Lanes 1–8 represent the amplification products of PCRs in which DNA isolated from single BFU-e colonies was used as template. The PCR amplification products were electrophoresed in an agarose gel and hybridized to a human γ-globin probe. The location of the expected 890-bp fragments is shown in the autoradiograph.

Gene Transfer into Peripheral Blood Cells

Although the therapeutic target cell for γ-globin gene expression is the red blood cell, the human γ-globin gene will be integrated in the genome of all the progeny of transduced hematopoietic stem cells. Thus, we used PCR analysis of nucleated peripheral blood cells as a surrogate assay for the transduction of nucleated red blood cell precursors in the bone marrow that give rise to circulating red blood cells. We performed semiquantitative PCR to look for the presence of human γ-globin gene sequences in peripheral blood DNA isolated from the four transplanted mice at monthly intervals beginning at one month following transplantation. By comparing the intensity of the hybridization signals to those obtained from several dilutions of DNA standards in

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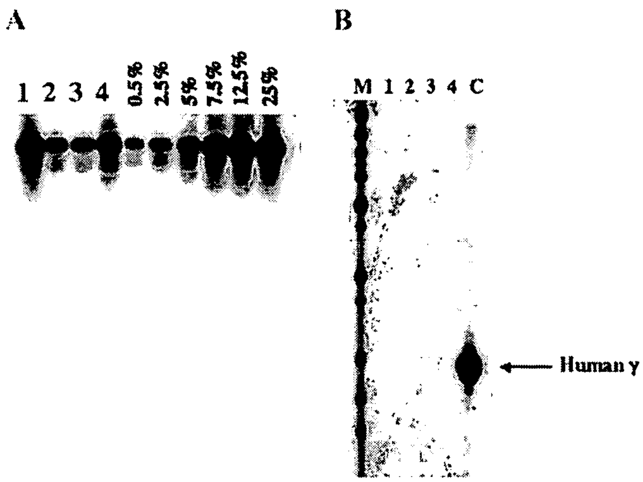

FIG. 3. Analysis of integration and expression of the human γ-globin gene in blood of transplanted mice. (A) An autoradiograph of a semiquantitative PCR assay to estimate the efficiency of integration of the human γ-globin gene in peripheral blood DNA of transplanted mice. The intensity of the PCR products in the transplanted mice DNA (lanes 1–4) is compared to the intensities of the PCR products in multiple dilutions (0.5 to 25%) of a murine DNA sample in which the human γ-globin gene is present at 1 copy/cell. (B) An autoradiograph representing an S1 assay for quantification of human γ-globin mRNA. In lanes 1–4, the assays included 2 μg of total RNA isolated from the peripheral blood of each of the transplanted mice. In lane 5, 5 μg of K562 RNA were used as a positive control.

which the human γ-globin gene is present at 1 copy/cell, we estimate that the efficiency of gene transfer in the four transplanted mice ranged from 2.5% to greater than 25% (Fig. 3). This relatively efficient gene transfer is consistent with the previously determined high titer of the retroviral stocks generated from the same producer cells (12).

Expression Analysis in Murine Peripheral Blood Cells

We performed quantitative S1 nuclease assay to quantify the expression of the human γ-globin gene in peripheral blood cells of the four transplanted mice 1 month following transplantation. As seen in Fig. 3, human γ-globin mRNA could not be detected in any of the transplanted mice (lanes 1–4) while it could easily be detected in lane C which consisted of 5 μg of total RNA isolated from K562 cells. S1 assays were performed once a month for 4 consecutive months using peripheral blood RNA that was harvested from all 4 transplanted mice. We failed to detect any expression of the human γ-globin gene in the peripheral blood of any of the 4 mice throughout this period (data not shown). We also could not detect the presence of human γ-globin chains by immunofluorescence in red blood cells from the 4 transplanted mice at any time during the course of the study (data not shown).

DISCUSSION

The early studies of the regulatory elements of the human globin genes were performed by transient transfections of globin gene expression constructs into HeLa cells or COS cells (22, 23). Although such studies were useful for the identification of the regulatory elements responsible for basal promoter activity, this approach was not suitable for the study of the regulatory elements responsible for the erythroid-specific and stage-specific expression of the human globin genes *in vivo*. In contrast, MEL cells which express murine adult-type β-globin in an inducible manner provided a convenient experimental system for the study of erythroid-specific and developmental stage-specific regulation of human globin gene expression (24, 25). More recently, the use of MEL cells in studies of the regulations of globin gene expression has been largely replaced by more physiologic transgenic mouse systems (26, 27). In contrast, MEL cells continue to be widely used for the pre-clinical testing of new retroviral β-globin gene transfer vectors (13, 28–30). In such studies, the level of expression of the human β-globin gene in retrovirally transduced MEL cells is highly predictive of its level of expression in mouse red blood cells *in vivo* following bone marrow transduction/transplantation (8–10). In studies from our own laboratory using HS-40-based β-globin retroviral vectors similar in design to the vector used in the experiments described above, we also observed a strong correlation between the level of expression of the human β-globin gene in MEL cells *in vitro* and in murine red blood cells *in vivo* (31). Thus, in spite of the availability of excellent animal models of bone

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marrow transplantation, MEL cells continue to serve as a simple and convenient surrogate system that accurately predicts the level of expression of the human β-globin genes in erythroid cells *in vivo*.

There has been no published experience that we are aware of that describes the use of γ-globin retroviral vectors in long term bone marrow transduction/transplantation experiments. In contrast to the experience with the human β-globin gene, the experiments described in this report demonstrate that MEL cells are clearly not suitable for the pre-clinical evaluation of γ-globin gene transfer vectors. Whereas the human γ-globin gene of the N2Aγ(HS-40) vector is expressed at high levels in MEL cells *in vitro* (12), it is completely silent in the adult erythroid environment of murine red blood cells *in vivo* (Fig. 3). It also appears that transgenic mice may not always be suitable for predicting expression of a γ-globin gene *in vivo* following retroviral gene transfer. Previous studies by Stamatoyannopoulos *et al.* showed that a γ-globin gene whose promoter is truncated to position −382 can be expressed at high levels in adult transgenic mice whereas a gene truncated to position −730 is appropriately silenced in adult erythroid cells (32). Since the γ-globin gene that was used in our retroviral vector was truncated to position −410 (12), we fully expected the gene to be expressed *in vivo* in adult erythroid cells. However, in spite of relatively efficient gene transfer reflected by the presence of the γ-globin gene in 2.5 to 25% of the circulating blood cells of the four transplanted mice, neither γ-globin mRNA nor γ-globin chains could be detected in the red blood cells of any of these mice.

Although it is theoretically possible that the lack of expression of the γ-globin gene may be a result of integration of the retroviral sequences in "nonpermissive" chromatin domains, we believe this is an unlikely explanation for the total lack of expression of the human γ-globin gene *in vivo*. First, when the efficiency of gene transfer is relatively high as in the experiments described above, retrovirally transduced circulating blood cells are likely to be derived from several transduced hematopoietic progenitor/stem cells rather than from a single transduced stem cell. Thus, to explain the complete absence of expression of the γ-globin gene in the 4 transplanted mice, all retroviral integration events in all 4 mice would have had to take place in non-permissive chromatin domains. Second, we had previously demonstrated that the human γ-globin gene of the N2Aγ(HS-40) vector is expressed in MEL cells at a remarkably uniform level regardless of the position of integration (12). As a matter of fact, we have never seen any integration event in MEL cells that resulted in absent or even low level of expression of the γ-globin gene (unpublished observations). Third, in similar *in vivo* gene transfer experiments in which we used a β-globin retroviral vector whose design is similar to N2Aγ(HS-40), we observed a strong correlation between the efficiency of retroviral integration and the level of expression in peripheral red blood cells with no evidence of position effects (31). Thus, we believe that the most likely explanation for the total lack of expression of the human γ-globin gene *in vivo* is that its proximal regulatory elements prevent its expression in the adult environment of murine red blood cells.

If the murine *in vivo* experiments described above are extrapolated to the human system, the N2Aγ(HS-40) vector that was used in this study would clearly not be suitable for the gene therapy of sickle cell disease or β-thalassemia. Previous studies in transgenic mice had shown that the replacement of the promoter of the human γ-globin gene with that of the human β-globin gene would allow the expression of γ-globin mRNA in adult cells *in vivo* (33). Thus, the replacement of the promoter of the human γ-globin gene with that of the human β-globin gene in the N2Aγ(HS-40) vector may allow fetal hemoglobin production *in vivo* in red blood cells of patients with sickle cell disease. This hypothesis should first be examined *in vivo* in the same murine model of bone marrow transplantation that was used in this report. The success of such an approach will make it possible to study the effects of high-level expression of the human γ-globin gene on the phenotype of sickle red blood cells in the murine models of sickle cell disease (34–36). This will set the stage for the use of such vectors for the gene therapy of patients with sickle cell disease.

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